556 PTEN can suppress NSCLC invasion through PI3K/AKT/NFkB pathway

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Background: It is now widely accepted that human carcinogenesis is a multi-step process and phenotypic changes during cancer progression reflect the sequential accumulation of genetic alteration in cells. Thus, in order to understand the process of acquisition of metastatic phenotypes in cancer cells, it is indispensable to identify genes whose alterations accumulate during cancer progression and correlate with metastatic phenotypes of cancer cells. For this reason researchers have been searching for genes that are preferentially altered in invasive & metastatic cancer cells and have activities to regulate their invasive & metastatic potentials. In non small cell lung cancer (NSCLC) PTEN/MMAC1 gene is frequently inactivated. However, it still remains unclear whether this gene is involved in the regulation of metastatic potential in lung cancer cells.
Material and Methods: PTEN cDNA isolated from cDNA library and then cloned tetracycline inducible vector. PTENG129R and PTENG129E mutants are established by site direct mutagenesis reaction. Stable transfection conformed by FUGEN HD and NFkB activity detected by using Lusiferase gene reporter system, expression levels of PTEN, Akt, Betacatenin and E-Cadherin were detected by western blot.
Results: Our data have shown that PTEN expression is very important for normal lung cells and a lot of lung cancer cell lines do not express the PTEN tumour suppressor gene, and there is a correlation between PTEN expression loss and increased AKT activity. We also found that stable wt PTEN expression, which was created stable transfection in no PTEN expressed NSCLC, is suppressed the cell invasion at 72%. For the determination of the mechanisms, we created PTENG129R and PTEN129E mutants vectors which are catalytically inactive and lipidphosphatase death PTENs respectively on tetracycline inducible vector. Constitutive expression of wild type PTEN, but not neither catalytically inactive mutant (PTENG129R) or Lipid phosphatase death mutant (PTENG129E) reduced phosphorylation on Ser-473 of AKT and the activation of NFkB, and the cellular invasion in NSCLC cell line PC14. We also find that only wt PTEN expression can induce beta catenin expression and reduce E-cadherin expression. Moreover we showed that only continuously AKT expression is enough for the induce invasion on NIH3T3 cells.
Conclusions: PTEN tumour suppressor gene product can control Akt and NFkB activation. Knowing that NFkB is a transcriptional activator which regulates survival pathways, and that metastasis is a process related with cell survival, one can comprehend the presence of a possible interaction between metastasis and NFkB activation. We conclude that PTEN is suffient to reduce the invasive potential of NSCLC cell invasion by lipid phosphatase activity and implies that PI3K/AKT/NFkB/Twist pathway is important to invasiveness on NSCLC.
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557 Myelin basic protein in malignant brain tumour − source of increased myelin basic protein in cerebrospinal fluid

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Background: We reported that the level of myelin basic protein (MBP) in the cerebrospinal fluid (CSF) increased and reflected the effect of treatment in malignant brain tumours (BTs) (Neurosurgery 34, 1994). We investigated the mechanism of the increased level of CSF MBP pathologically using clinical specimens and experimental BTs under the permission of the Ethics Committee of the Nozaki Tokushukai Hospital.
Material and Methods: Surgical specimens of glioma, metastatic brain tumour (MBT), brain tissue adjacent to MBTs, other BTs and cyst fluid of various kinds of BTs were examined by radioimmunoassay. The immuno-histochemical localization of MBP in BTs was also examined and compared with those of glial fibrillary acidic protein (GFAP) and 2′,3′-cyclic nucleotide, 3′-phosphodiesterase (CNP). Moreover, immuno-histochemically double-stained specimens using mouse monoclonal anti-GFAP and rabbit polyclonal anti-MBP antibodies were subjected to laser microscopy to confirm the sources of MBP in BT tissue. Finally, MBTs and glioblastomas were investigated by electron microscopy.
Results: Malignant glioma cyst fluid showed the highest levels of MBP among the BTs examined. However, the quantitative measurements of tissue MBP showed the highest levels in the brain surrounding MBTs. Immuno-histochemical analysis of MBP in glioma showed marked staining in the brain at the border zone where reactive astrocytes were observed. GFAP immunohistochemistry also showed marked staining in the same region, while staining for CNP was weak. In MBTs, areas of normal brain tissue close to tumour showed marked GFAP staining, and MBP and CNP staining were confirmed in areas close to the strongly GFAP-immunolabeled regions. On electron microscopic analysis in the brain tissue adjacent to tumour, degenerated myelin due to massive edema fluid and phagocytosis of degenerated myelin by macrophage or microglia in MBT, and oligodendrocytes surrounded by many myelin as well as degenerated myelin in glioblastoma were observed.
Conclusion: From these findings, we speculated that MBP is produced by oligodendrocytes around tumour cells. On the other hand, MBP is released from injured oligodendrocytes in the process of degeneration by compression of tumour cells or edema fluid. We also speculate that the increased MBP in the cerebrospinal fluid was due to induction of release from microglia and macrophages by phagocytosis of degenerated myelin.

558 Epstein Barr virus latent membrane protein 1 in Hodgkin lymphoma

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Background: Epstein–Barr virus (EBV) is now known to be firmly associated with Hodgkin lymphoma (HL) and is detected in more than 50% of patients. Studies have shown that EBV-positive Reed-Sternberg cells express the latent membrane proteins (LMPs) which possess oncogenic properties and may lead to the activation of signalling pathways like MAP kinases (MAPK) and phosphatidylinositol-3 kinases (PI3K). Alterations of these signalling pathways in the presence or absence of viral factors, such as LMPs have not been reported in Hodgkin's lymphomas. The working hypothesis of the project is that LMP1 expression in lymphocytes will lead to the activation of the MAPK and PI3K pathways which are associated with the pathogenesis of HL.
Material and Methods: Plasmid harbouring LMP1 and empty vector was transfected into Hodgkin lymphoma cell line. A small volume of cells was harvested to check the transfection efficiency by flow cytometer and Western blot analysis to confirm the specificity of the expressed protein. A sample of cell lysate protein was subjected to SDS-PAGE before blotting onto nitrocellulose membrane to detect and quantify the phosphorylated status of major proteins involved in MAPK and PI3K pathways. Immunohistochemistry was performed on HL EBV-positive and HL EBV-negative tissues to show the immunophenotype changes, cytoplasmic and/or nuclear localization of activated MAPK and PI3K pathways.
Results: The ERK, JNK, p38 MAPK and Akt pathways were constitutively activated in Hodgkin cell lines and RS cells of Hodgkin tissues. Although p44 and p42 are both expressed at high levels in L428, L591 and KMH2 cells, only p42 was strongly phosphorylated in KMH2-EBV cells. C-terminal activation regions of LMP1 play an important role in activating both JNK and p38 MAPK pathways. We found isoforms JNK2 and JNK3 were highly expressed in all cell lines as compared to JNK1. However, JNK1 and JNK2 were detected in both nucleus and cytoplasm of RS cells of all tissues. Our study showed that phosphorylation of JNK was driven by LMP1. It was reported that JNK appears to have a unique prosurvival role. We reported that from the immunohistochemistry analysis, p38 MAPK was dual-phosphorylated at Thr180/Tyr182 and subsequently activated ATF-2. Akt2 which stimulates cell motility was expressed in all cell lines and tissues compared to Akt1 and Akt3. We believe differential regulation of Akt isoforms must be functionally important in the biology and survival signalling of RS cells. Phosphorylation of Akt at Ser473 was also highly expressed in tissues and KMH2-EBV but not in L428 cells. We found that isoform GSK3α was instead abundantly expressed and activated in Hodgkin cell lines especially KMH2-EBV and tissues.
Conclusion: We suggest that these signalling cascades are involved in the pathogenesis of Hodgkin lymphoma. Collectively, our data demonstrate that EBV LMP1 play an important role in activation of ERK, JNK, p38 MAPK and Akt pathways to maintain its survivability in Hodgkin tumours.

559 Galectin-1 expression in Hodgkin-lymphoma cells

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Background: Galectin-1 (gal-1) is a carbohydrate-binding protein, a regulator of immune responses and T-cell homeostasis. The mechanisms modulating gal-1 expression are still largely unknown. Gal-1 expression or overexpression was observed in several human tumours. The aim of our study was to examine gal-1 expression in different Hodgkin-lymphoma (HL) samples – both in tumour cells and in the stroma –, and to determine regulator T-cell phenotype in the microenvironment. Furthermore *in vitro* and *in vivo* models were initiated for the investigation of gal-1 expression after treatments.
Materials and Methods: The expression of different proteins was examined in tumour cells and in their microenvironment in formalin fixed and paraffin embedded HL samples (from 93 patients) by immunohistochemistry (IHC) (gal-1, CD4, Foxp3). Gal-1 was examined in several lymphoma/leukemia cell lines by immunocytochemistry (ICC). A HL cell line (KMH2) was cultured and treated *in vitro* and xenotransplanted into SCID mice. The effect of several

cytokines and chemotherapeutic agents on growth and gal-1 expression was explored in tumour cells and xenografts by Western-blotting, IHC/ICC, real-time PCR and FACS.

Results: Gal-1 overexpression was detected in HL cells and also in the extracellular matrix of HL, especially in the nodular sclerosis subtype. Gal-1 expression and regulatory T-cell phenotype were observed in corresponding regions in different HL subtypes. Lymphoma/leukaemia cell lines expressed different levels of gal-1 (from zero to high). Gal-1 was overexpressed in the KMH2 cell line and xenograft. Treatments (mycophenolate-mofetil, rapalogs, TGFb, PI3K-inhibitor, cyclophosphamide) have been initiated in *in vitro* and *in vivo* HL models in order to study their effect on proliferation and gal-1 expression.

Conclusion: Our data suggests that gal-1 overexpression may be of importance in Hodgkin-lymphoma, presumably by promoting the survival of tumour cells. Certain subtypes of Hodgkin-lymphomas contain relatively few tumour cells compared to the microenvironment, which suggests a remarkable interaction between these components, perhaps modulated by gal-1. Therefore, a more thorough knowledge of the regulation of gal-1 expression and function is required using HL-samples, *in vitro* and *in vivo* HL-models.

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560 BRCA1 protein expression correlates with cancer stem cell markers in primary breast cancer

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Background: Based on Cancer Stem cell (CSC) hypothesis cancers originate in tissue stem or progenitor cells through the dysregulation of self-renewal process. Breast cancer cells with a $CD44^+/CD24^{-/low}$ phenotype have been proposed to have tumour initiating properties with stem cell-like and invasive features. BRCA1is an important susceptibility gene for breast cancer which plays a crucial role in DNA repair, activation of cell-cycle checkpoints, and maintenance of chromosome stability. The clinical, molecular, and pathologic features of breast cancer in BRCA1 mutation carriers suggest that BRCA1 may function as a stem-cell regulator.

Material and Methods: The purpose of the current study was to investigate the relationship between BRCA1 protein expression and clinicopathological characteristics, and putative cancer stem cell markers in a well-characterized series of unselected breast carcinomas. Immunohistochemistry was performed on 156 primary operable breast tumours using a monoclonal anti-BRCA1 primary antibody.

Results: Adjacent normal breast tissue showed strong BRCA1 immunoreactivity mainly localized to nuclei of the cells with no cytoplasmic staining. In breast cancers, complete loss of nuclear expression was observed in 23 cases (15%), whereas cytoplasmic expression was found in 133 breast carcinomas (85%) which was correlated with nuclear pattern. Absent or reduced nuclear BRCA1 expression was observed more frequently in invasive ductal carcinoma, and less frequently in medulary carcinoma and lobular carcinomas. (p-value = 0.005).

Altered BRCA1 expression was significantly associated with high grade and poor prognosis breast tumours (p value = 0.006). It was also more often seen in early onset breast cancer patients ($\leqslant$40 years) rather than patients over age of 40 (p value = 0.04). We further established a significant correlation between the BRCA1 expression levels and the CD44+/CD24− cancer stem cell phenotype in primary breast tumours (P = 0.02).

Conclusion: Taken together, our results indicate that loss of BRCA1 expression is a marker of tumour aggressiveness, potentially linked to BRCA1 status and a cancer stem cell phenotype in primary breast cancer. Breast cancer stem cells are more likely than non stem cell to have low levels of BRCA1 expression. These finding support the idea that loss of BRCA1 expression may result in an accumulation of genetically unstable breast stem cells, providing targets for further carcinogenic events.

561 Renin-angiotensin system expression in myeloproliferative diseases

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Myeloproliferative diseases present the group of clonal malignant diseases of hematopoietic stem cell. Somatic mutation of *JAK2* gene ($JAK2V617F^+$) is present in most of the patients (>90%) with polycythemia vera (PV), and 50% of patients with essential thrombocythemia (ET). This mutation causes the constitutive activation of tyrosine kinase and the consequence is cytokine independent proliferation of cells. Signaling pathway JAK2/STAT5/Bcl-xL is essential for erythropoiesis, controlling cell proliferation and survival. In $JAK2V617F^+$ PV and ET, growth of erythroid progenitors is erythropoietin independent. There is a lot of evidence of local renin-angiotensin system (RAS) presence in bone marrow affecting cell proliferation and differentiation. There is an increase of mRNA expression of angiotensinogen (AGT), rennin (REN) and angiotensin II receptor 1 (AT2R1) in bone marrow of $JAK2V617F^+$ PV and ET patients. Our research is focused on understanding the correlation of these two pathways in order to find the control points that can be used as a drug targets for myeloproliferative disorders.

Bone marrow mononuclear cells from PV and ET patients were seeded on MethoCult (StemCell) in medium with and without erythropoietin (EPO). At day 13 erythroid colonies were observed for morphology differences, colony density and isolation of DNA, RNA and proteins (Trizol, Invitrogene). DNA is used to determine JAK2 status by allele-specific PCR, RNA for real-time PCR detection of RAS components and proteins for Western detection of AT2R1 protein.

We analyzed 5 different bone marrow samples by now – 3 PV ($JAK2V617F^+$), 1 PV ($JAK2V617F^-$) and 1 ET ($JAK2V617F^+$). $JAK2V617F^+$ PV and ET erythroid colonies grown without EPO were smaller (50–100 cells), paler and in lower density then erythroid colonies grown with EPO (>200 cells). No erythroid colonies were noticed in $JAK2V617F^-$ PV sample without EPO. We collected high quality DNA, RNA and protein from cca 10^4 cells. We modified protein extraction method to gain better solubilization of proteins by resuspending them in 2% DEA. Preliminary results suggest increase of AT2R1 expression in $JAK2V617F^+$ patients when compared with $JAK2V617F^-$ patients. Western and RT-PCR data are in preparation.

Confirmation of higher expression of RAS components in patients that have constitutive activation of JAK2 will enable us to use drugs like ACE inhibitors and AT2R1 antagonists in assessing erythroid proliferation-differentiation process.

562 Breakpoints at 17p11.2 detected by high-resolution 500K SNP arrays identifies most metastatic colorectal carcinomas

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Background: The genetics of metastatic colorectal cancer have been studied for many years using a variety of techniques with limited resolution which hampers identification of specific underlying cancer-associated genes. Introduction of high-density single nucleotide polymorphism arrays has allowed identification of small regions of chromosomal gains and losses with a much higher resolution of down to 2.5 kb.

Material and Methods: Here we used 500K SNP mapping arrays to map the overall genetic lesions present at diagnosis in 23 primary sporadic CRC patients with liver metastasis. In order to evaluate the consistency of the chromosomal changes identified by the SNP-arrays, interphase FISH analysis were performed in parallel for a total of 24 chromosome regions from 20 different chromosomes.

Results: The highest frequency of copy number (CN) losses detected corresponded to chromosomes 1p (n = 17; 74%), 8p (n = 18; 78%), 14q (n = 15; 65%), 17p (n = 19; 83%), 18 (n = 21; 91%) and 22q (n = 17; 74%) while CN gains more frequently involved chromosomes 1q (n = 10; 43%), 7 (n = 20; 87%), 8q (n = 17; 74%), 13q (n = 18; 78%), 20q (n = 20; 87%) and X (n = 13; 57%). SNP arrays allowed the identification of small (<1.3 Mb) and extensive/large (>1.5 Mb) altered DNA sequences. Interestingly, several of these regions contain cancer genes known to be involved in colorectal cancer and metastatic process (particularly among the amplified chromosome regions).

Conclusions: Overall our results showed a high degree of correlation between both methods, including for the most frequently altered regions. Moreover, four recurrent chromosomal breakpoints were identified at chromosome 1p12, 8p12, 17p11.2 and 20p12.1. Interestingly, detailed analysis of recurrent chromosomal breakpoints reveals a highly prevalent breakpoint at 17p11.2 which may target genes such as the *FAM27* gene whose role in the disease deserves further investigation.